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A Mechanism to Alter Reversibly the Oligomeric State of a Membrane-bound Protein Demonstrated with *Escherichia coli* EII^{mtl} in Solution*

(Received for publication, August 13, 1997, and in revised form, November 10, 1997)

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This paper reports that the aggregation state of a membrane protein can be changed reversibly without the use of chaotropic agents or denaturants by altering the attractive interactions between micelles of polyethylene glycol-based detergents. This has been documented using mannitol permease of *Escherichia coli* (EII^{mtl}), a protein whose activity is dependent on the dimerization of its membrane-embedded domains. We show that the driving force for the hydrophobic interactions responsible for the dimerization can be decreased by bringing the protein into a less polar environment. This can be done simply and reversibly by increasing the micelle cluster size of the solubilizing detergent since the micropolarity in the micelle decreases upon clustering and is directly related to the cluster size.

The micelle cluster size was varied at a fixed temperature by adding sodium phosphate or a second detergent with a distinct clustering behavior, and the changes were quantified by quasi-elastic light scattering and by determining the cloud point or demixing temperature (T_d) of the detergent. Maximal EII^{mtl} activity was found when no micelle clustering occurred, but the activity gradually decreased down to 5% of the maximal activity with increasing cluster size. The inactivation was found to be completely reversible. The kinetics of heterodimer formation were also significantly affected by changes in the micelle cluster size as expected. Increasing the cluster size resulted in faster formation of functional heterodimers by increasing the rate of homodimer dissociation. This phenomenon should be generally applicable to controlling the oligomeric state of membrane-bound proteins or even water-soluble proteins if their subunit association is dominated by hydrophobic forces.

It is well documented that the micropolarity of PEG-based[1] detergent micelles decreases when they form clusters and that this decrease is directly related to the micelle cluster size (1–3). In principle, this decrease in micropolarity should be able to be used to transfer a membrane protein, solubilized by such a detergent, to a less polar environment. Only PEG-based detergents show this clustering behavior. It can be induced by heating where, at a certain temperature, the cloud point or demixing temperature (T_d), the cluster size reaches a value which initiates separation of the micellar phase into two new micellar phases, one consisting of the micellar clusters and one containing a low concentration of micelles (2). Phase separation or demixing of PEG-based detergents into two new micellar phases at T_d upon heating has been studied in detail with various techniques such as light, neutron and x-ray scattering, viscosity measurements, and NMR spin lattice relaxation (2, 4–8). Such studies suggest that the intermicellar forces become stronger upon heating due to a decrease of the hydration of the PEG chains. This leads to stronger van der Waals interactions between the micelles and the formation of micelle clusters. It is believed that the size of the micelle does not change upon heating (7), but the size of the clusters increases asymtotically and follows a power-law given by $(T_d - T)/T_d$ (4, 6). The attractive forces between micelles can also be increased at a fixed temperature, T, by the addition of certain inorganic salts, especially phosphate or fluoride salts, or by mixing with a detergent with a lower T_d (2). Whether one increases T or lowers T_d, the result in the same, the interval $T_d - T$ decreases, and as the two approach one another, the micelle cluster size increases until, at $T = T_d$, phase separation occurs. This demixing property has been used in biochemical studies to separate hydrophobic proteins from more hydrophilic ones (9). Hydrophobic proteins concentrate in the detergent-rich phase upon heating the detergent solution above T_d. After centrifugation, this phase can be easily separated from the aqueous phase, containing the hydrophilic proteins. Triton X-114 with T_d = 22 °C is often used in such procedures.

Here we demonstrate that these same intermicellar attractive forces can be used in a more subtle way to disrupt the hydrophobic forces responsible for subunit interactions and thereby reversibly control the association state of EII^{mtl} in PEG-based detergents. EII^{mtl} is inactive as a monomer and phophorylates mannitol when in the dimeric state. Hydrophobic forces are involved in the dimerization process (10). Both the activity of EII^{mtl} and the rate of formation of EII^{mtl} heterodimers can be controlled by choosing a specific micellar cluster size. The results are explained by relating the decrease

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[1] PEG, polyethylene glycol; EII^{mtl}, mannitol permease of *E. coli*; HPr, histidine-containing protein; EI, enzyme I of the phosphoenolpyruvate-dependent carbohydrate transport system; mtl, mannitol; mtl-P, mannitol-1-phosphate; DTT, dithiothreitol; PEP, phosphoenolpyruvate; dPEG, decylpolyethylene glycol 300; $C_{10}E_4$, decyltetra(ethylene glycol); $C_{10}E_5$, decylpenta(ethylene glycol); $C_{10}E_6$, decylhexa(ethylene glycol); dMal, *n*-decyl β-D-maltopyranoside; R_h, apparent hydrodynamic radii; T_d, cloud point or demixing temperature; P-EII^{mtl}, phosphorylated EII^{mtl}.

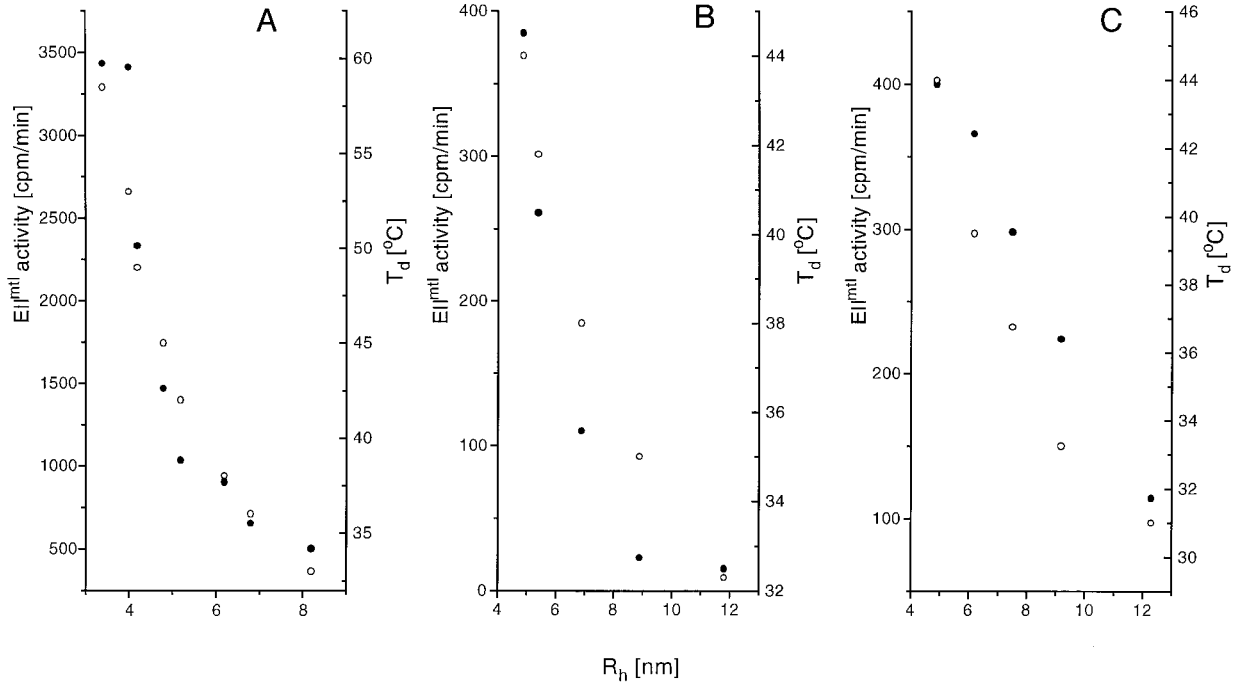

FIG. 1. **Relationship between the cloud point (T_d) and apparent hydrodynamic radii (R_h) of solution containing dPEG or $C_{10}E_5$ and the PEP-dependent phosphorylation activity of EII^{mtl} in these solutions.** R_h measurements were performed at 30 °C on a solution containing 25 mM Tris·HCl, pH 7.6, 5 mM DTT, 5 mM $MgCl_2$, 5 mM PEP, 0.25% (v/v) detergent, and eventually Na_3PO_4 or $C_{10}E_4$ to lower the T_d. The T_d measurements were performed on the same solutions. The PEP-dependent phosphorylation activity of EII^{mtl} was determined by incubating the enzyme for 5–10 min at 30 °C in 25 mM Tris·HCl, pH 7.6, 5 mM DTT, 5 mM $MgCl_2$, 5 mM PEP, 0.25% (v/v) detergent, 20 μM HPr, 0.33 μM EI, and eventually Na_3PO_4 or $C_{10}E_4$ to lower the T_d. The reaction was started after the incubation period by adding [^{3}H] mannitol with a final concentration of 60 μM (final volume 100 μl). At four different times, 20 μl-aliquots were taken, and the amount of mannitol-1-phosphate was quantified. *A*, relationship between R_h and T_d (○) and the activity of EII^{mtl} (1.0 nM) *versus* R_h in these buffers with 0.25% dPEG and various amounts of Na_3PO_4 to lower the T_d (●). The T_d decreased linearly from 58 °C at 0 mM Na_3PO_4 to 33 °C at 250 mM. *B*, relationship between R_h and T_d (○) and the activity of EII^{mtl} (0.08 nM) *versus* R_h in buffer with 0.25% $C_{10}E_5$ and various concentrations of Na_3PO_4 to lower the T_d (●). The T_d decreased linearly from 44 °C at 0 mM to 31 °C at 250 mM Na_3PO_4. *C*, relationship between R_h and T_d (○) and the activity of EII^{mtl} (0.08 nM) *versus* R_h in buffer with 0.25% pure $C_{10}E_5$ or a mixture of $C_{10}E_5$/$C_{10}E_4$ (●). The T_d was lowered by increasing the percentage of $C_{10}E_4$. The T_d decreased linearly from 44 °C for pure $C_{10}E_5$ to 31 °C for 60% $C_{10}E_5$, 40% $C_{10}E_4$ (v/v). The specific enzyme activities under these conditions in the absence of Na_3PO_4 or $C_{10}E_4$ were 2270, 2965, and 3090 nM mannitol-1-phosphate/min/nM enzyme in Fig. 1, *A*, *B*, and *C*, respectively.

in micropolarity of the micelles upon clustering with the decrease in the driving force for hydrophobic bonding, the interactions responsible for EII^{mtl} dimerization.

EXPERIMENTAL PROCEDURES

Materials—dPEG, $C_{10}E_6$, $C_{10}E_5$, and $C_{10}E_4$ were supplied by B. Kwant (Kwant High Vacuum Oil Recycling and Synthesis, Bedum, The Netherlands). dMal was from Sigma. Na_3PO_4 solutions were adjusted to pH = 7.6 with concentrated H_3PO_4.

Q-Sepharose Fast Flow and S-Sepharose Fast Flow were from Pharmacia (Sweden); hexyl-agarose was from Sigma. D-[1-^{3}H]Mannitol (976.8 GBq/mmol) was from NEN Life Science Products. EI and HPr were purified as described previously (11–13). All other reagents were analytical grade. The purification of EII^{mtl} was as described previously for EII^{mtl}(C384S) (14).

Mannitol Phosphorylation Assays—The PEP-dependent mannitol phosphorylation activity of EII^{mtl} was measured as described (15). The assay buffer contained 25 mM Tris·HCl, pH 7.6, 5 mM DTT, 5 mM $MgCl_2$, 5 mM PEP, 0.25% (v/v) detergent, 20 μM HPr, and 0.33 μM EI. The reaction was started with 60 μM [^{3}H]mannitol (1 mM for the experiments presented in Fig. 2). Concentrated stock solutions (1–2 μM) of EII^{mtl} were used, which were diluted 1,000-fold when the enzyme was assayed. Therefore, the influence of the dPEG (T_d = 58 °C) in this stock solution during the assay conditions can be neglected. All reactions were performed at 30 °C in buffers with T_d > 30 °C. Changes in T_d (and thus R_h) are known to occur abruptly (7) as was checked under the conditions used in this study.

Concentration Determinations on EII^{mtl} Samples—The EII^{mtl} concentrations were determined by flow dialysis which quantitates the number of mannitol binding sites (16), assuming one high affinity binding site (K_D ~100 nM) per EII^{mtl} dimer in accordance with the observations of Pas *et al.* (14).

Light Scattering Experiments—Light scattering experiments were performed at 30 °C by using a DynaPro-801TC instrument (Protein Solutions Inc., Charlottesville, VA), equipped with a thermostated cell. Detergent (0.25%, v/v) was dissolved in 25 mM Tris·HCl, pH 7.6, 5 mM DTT, 5 mM $MgCl_2$, and 5 mM PEP. In one case, 0.5% dPEG was used since no stable signal at 0.25% was observed. Solutions were filtered through 0.1 μm Anotop10 filters (Whatman). Data were analyzed using the software supplied by the manufacturer. Data could be resolved by the theoretical single exponential autocorrelation function (monomodal analysis), indicating that the solutions were monodisperse. Each sample was measured at least seven times. Standard deviations in R_h were 0.1 nm or less.

Cloud points (± 0.5 °C) were determined by heating a detergent solution in a test tube with a thermometer.

RESULTS

Effect of Micelle Cluster Size on the Activity of EII^{mtl}—Micelles clustering is reflected in an increase in the apparent hydrodynamic radius (R_h) of the micelle, and this occurs as the temperature of the solution approaches the cloud point (T_d). The phosphorylation activity of EII^{mtl} has been determined at 30 °C in PEG-based detergent mixtures with various cloud points, and the apparent hydrodynamic radii of the micelle clusters of these solutions under the assay conditions has been determined by quasi-elastic light scattering experiments. The T_d of PEG-based detergents was lowered by increasing the concentration of sodium phosphate. Addition of up to 250 mM Na_3PO_4 (pH = 7.6) lowered the T_d of dPEG from 58 to 33 °C with a corresponding increase in R_h from 3.4 to 8.2 nm (Fig. 1*A*, ○). A plot of the enzyme activity against R_h shows that increased values of R_h result in a lowering of the enzyme activity down to 10% of the activity found in dPEG with R_h = 3.4 nm

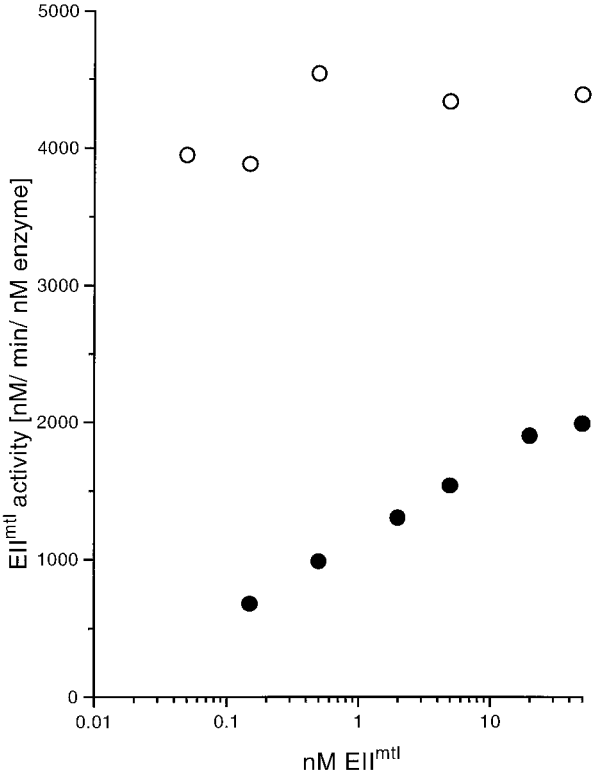

FIG. 2. **Relationship between the EII^{mtl} concentration and the PEP-dependent specific phosphorylation activity of EII^{mtl} in assay buffer with various T_d.** The specific activity of EII^{mtl} was determined at 30 °C by incubating the enzyme for 5–10 min in 25 mM Tris·HCl, pH 7.6, 5 mM DTT, 5 mM $MgCl_2$, 5 mM PEP, 0.25% dPEG (v/v), 20 μM HPr, 0.33 μM EI, and 200 mM (●) or 0 mM Na_3PO_4 (○). T_d is 38 and 58 °C, respectively. The reaction was started after the incubation period with 1 mM mannitol.

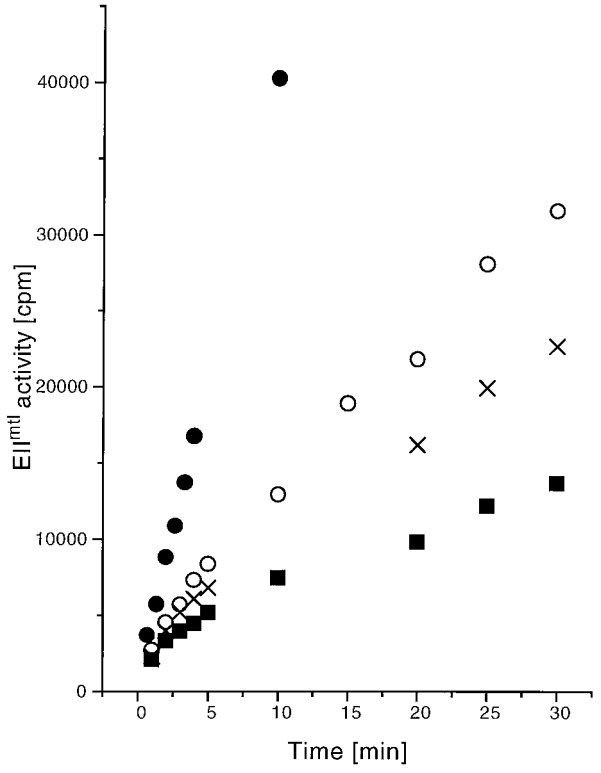

FIG. 3. **Change in PEP-dependent phosphorylation activity of EII^{mtl} upon a rapid change in apparent hydrodynamic radii (R_h) of the assay buffer.** 1.4 nM EII^{mtl} was incubated at 30 °C in 25 mM Tris·HCl, pH 7.6, 5 mM DTT, 5 mM $MgCl_2$, 5 mM PEP, 0.25% (v/v) dPEG (T_d = 58 °C), 20 μM HPr, and 0.33 μM EI for 5 min. The reaction was started after a 1.4-fold dilution in a solution containing mannitol and Na_3PO_4 resulting in final Na_3PO_4 concentrations of 0 (●), 215 (○), 235 (×), and 250 mM (■). The R_h values were 3.4, 6.4, 7.3, and 8.2 nm, respectively, and the corresponding T_d were 58, 37, 35, and 33 °C. Final mannitol concentration was 60 μM.

(Fig. 1*A*, ●). The activity in the latter buffer corresponds with the highest specific activity found for EII^{mtl} solubilized in a detergent (2000–3000 nM/min/nM enzyme when assayed with 60 μM mannitol). Increasing the dPEG concentration from 0.25 to 1% at 200 mM Na_3PO_4 did not change the EII^{mtl} activity (not shown). Fig. 1*B* shows that the sensitivity of the EII^{mtl} activity to a change in T_d was not only observed in the polydisperse detergent, dPEG, but also in the monodisperse PEG-based detergent, $C_{10}E_5$. The activity of EII^{mtl} in buffer containing pure $C_{10}E_5$ (T_d = 44 °C) was the same as that found in dPEG with T_d = 58 °C (17). Lowering the T_d via the introduction of Na_3PO_4 again resulted in increased values of R_h and a lowering of the EII^{mtl} activity. Finally, the T_d of these solutions could also be changed by mixing with another PEG-based detergent with a different T_d rather than with Na_3PO_4. Fig. 1*C* shows that mixing $C_{10}E_5$ (T_d = 44 °C) with $C_{10}E_4$ (T_d = 16 °C) also resulted in an increase in R_h and a decrease in EII^{mtl} activity.

The decreasing EII^{mtl} activity with increasing R_h should be reflecting the change in oligomerization state of EII^{mtl} from active dimers into less active or inactive monomers. This is supported by the behavior of the specific activity of EII^{mtl} as a function of the enzyme concentration in buffer containing detergent with high (Fig. 2,) ○) and low (●) T_d. These experiments were performed under V_{max} conditions (1 mM mannitol instead of 60 μM), which allowed the enzyme to be assayed between 0.05 and 50 nM at a single mannitol concentration. In dPEG with a T_d = 58 °C (Fig. 2, ○), an activity of ± 4000 nM/min/nM enzyme was found that was constant between 0.05–5 nM EII^{mtl}. In dPEG buffer with 200 mM Na_3PO_4 (T_d = 35 °C) (Fig. 2, ●), the specific activity increased from less than 670 nM/min/nM enzyme at 0.05 nM enzyme to 2000 nM/min/nM enzyme at 50 nM EII^{mtl}, confirming that lowering the T_d results in dissociation of active dimers into less active or inactive monomers. Increasing the EII^{mtl} concentration shifts, by mass action, the equilibrium back to the active, dimeric form.

The reversibility of the change in association state upon a change in T_d has been demonstrated in $C_{10}E_5$ buffer. EII^{mtl} (0.8 nM) was incubated for 10 min at 30 °C in buffer containing 120 mM Na_3PO_4 (T_d = 34 °C) to cause the protein to dissociate. One portion of this solution was then diluted 10-fold with assay buffer containing the same Na_3PO_4 concentration while another portion was diluted with assay buffer lacking Na_3PO_4. After 10 min at 30 °C, the phosphorylation reaction was started by adding 60 μM mannitol. As expected, a low activity (240 nM/min/nM enzyme) was found for the enzyme incubated with buffer containing 120 mM Na_3PO_4 and diluted into the same buffer. A high activity (2055 nM/min/nM enzyme) was observed for the enzyme incubated with buffer containing 120 mM Na_3PO_4 but then diluted with the low salt buffer. The same high activity was measured for the control enzyme that had not been exposed to high Na_3PO_4 in the incubation or dilution phase. Therefore, the effect of a variation in T_d on the activity of EII^{mtl} is completely reversible.

Fig. 3 presents the effect of a rapid change in T_d on the EII^{mtl} activity. The enzyme was preincubated in a dPEG buffer with high T_d (58 °C) along with PEP, E1, and HPr to generate phosphorylated EII^{mtl} (P-EII^{mtl}) and then diluted into buffers with mannitol and dPEG with the same or lower T_d. The control (Fig. 3, ●) diluted into dPEG buffer with a T_d of 58 °C showed a rapid linear increase of mtl-P with time. But when

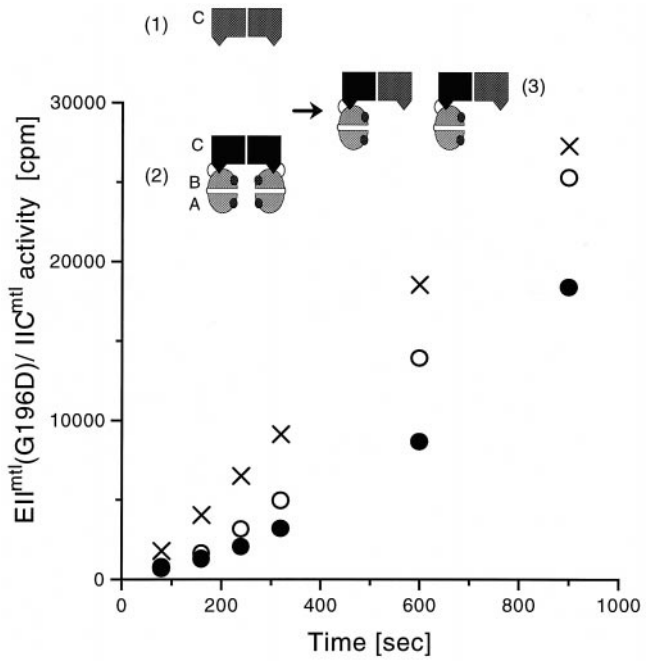

FIG. 4. **Dependence of the rate of appearance of the phosphorylation activity of EII^{mtl} (G196D)/IIC^{mtl} heterodimers on the apparent hydrodynamic radii (R_h) of the buffer.** EII^{mtl}(G196D) (20 nM) and IIC^{mtl} (100 nM) were incubated at 30 °C for 10 min in buffer containing 25 mM Tris·HCl, pH 7.6, 5 mM DTT, 5 mM $MgCl_2$, 5 mM PEP, 0.25% (v/v) dPEG, 20 μM HPr, 0.33 μM EI, and 0 mM (●), 90 mM (○), or 235 mM Na_3PO_4 (×) with T_d = 58, 50, and 35 °C corresponding to R_h = 3.4, 4.1, and 7.3 nm, respectively. The reaction was started with 60 μM mannitol. *Inset*, schematic representation of the complementation of IIC^{mtl} (*1*) and EII^{mtl}(G196D) (*2*) yielding EII^{mtl} (G196D)/IIC^{mtl} heterodimers (3). The nomenclature of the EII^{mtl} domains (A, B, C) has been used.

the samples were diluted into dPEG buffers with a lower T_d, the time dependence showed two phases. (i) In the first 4–5 min, the specific activity gradually decreases. (ii) After 5 min, the specific activity was stable, the lower the T_d then the lower the activity.

Since the new T_d values were reached immediately after mixing (see "Experimental Procedures"), these phases most likely reflect the time-dependent changes in the association state of the enzyme. During the first phase, the concentration of monomer in solutions with lower T_d increases until a new monomer-dimer equilibrium establishes itself. After 5 min, the new equilibrium is established, and the rates are linear with time. The results presented so far show a reversible dissociation of active EII^{mtl} dimers into less active monomers upon increasing R_h or correspondingly decreasing T_d. In the next section, the effect of variation of T_d on the formation of heterodimers will be presented.

Effect of Variation in Micelle Cluster ;size in Complementation Assays—The influence of micelle cluster size on the kinetics of heterodimer formation of EII^{mtl} has been studied by using two EII^{mtl} mutants, each of which lack PEP-dependent activity but become active upon formation of a heterodimer (complementation assay, see Fig. 4, *inset*). One such couple is EII^{mtl} (G196D), with drastically reduced binding affinity for mannitol, and IIC^{mtl}, [2] which is inactive by virtue of the missing A and B domains (18). Upon formation of a heterodimer, the phosphoryl group can proceed from PEP via HPr and E1 and the A and B domains of EII^{mtl} to mannitol bound in the C domain (see "Discussion"). Fig. 4 follows the rate of appearance of phosphorylation activity when these two enzymes are incubated in detergent of varying T_d. EII^{mtl} (G196D) and IIC^{mtl} were incubated for 10 min in phosphorylation buffer with dPEG and various concentrations of Na_3PO_4 shifting the T_d between 58 and 35 °C (R_h = 3.4–7.3 nm). The reaction was started with mannitol, and the accumulation of mannitol-1-phosphate over time was monitored. In detergent solutions with a high T_d (58 °C), a slow initial activity was observed that gradually increased in time and then became constant (Fig. 4, ●). When the T_d was decreased, initial activities increased, resulting in faster attainment of a constant PEP-dependent phosphorylation activity (○). In a detergent solution with a T_d of 35 °C, a high and constant activity was observed immediately (Fig. 4, ×). These results suggest a more rapid formation of functional heterodimers from inactive homodimers in detergent solutions with a lower T_d.

The following experiments were performed to examine the formation of heterodimers in more detail (Fig. 5). EII^{mtl} (G196D) and IIC^{mtl} were incubated for 10 min in buffer containing 25 mM Na_3PO_4, 5 mM DTT, and 0.25% dPEG (T_d = 58 °C). This solution was diluted 20-fold in the same buffer that now contained 5 mM $MgCl_2$ and 5 mM PEP, 0.33 μM EI, and 20 μM HPr for an additional 5 min to generate P-EII^{mtl}. Upon addition of mannitol, a low initial activity was found, which gradually increased with time (Fig. 5*A*, ●). But if the initial solution was diluted in buffer with high Na_3PO_4 to produce a low T_d (37 °C), maximal phosphorylation activity was observed immediately (Fig. 5*A*, ×). Similarly, if the incubation was performed under low T_d conditions and the assay at either high or low T_d conditions, maximal initial activity was found immediately (Fig. 5*A*, ○ and ■, respectively). Therefore, as long as both enzymes have been incubated with each other under low T_d conditions, formation of heterodimers is complete within the 5-min incubation period and does not reverse when the T_d is subsequently increased. Although more inactive monomers are expected in detergent solutions with a low T_d (Figs. 1 and 3), the high protein concentration used in these experiments will result in high heterodimer concentrations. These experiments show that formation of heterodimers is very fast when the T_d of the buffer is low; moreover, the specific activity of the heterodimer is not affected at high enzyme concentrations when the T_d is varied between 37 and 63 °C (R_h = 6.4–3.4). Incubation of EII^{mtl} with mannitol has been reported to stimulate the dissociation of EII^{mtl} dimers (19) and could also be expected to stimulate heterodimer formation. To test this, EII^{mtl} (G196D) and IIC^{mtl} were incubated for 10 min at 30 °C in assay buffer with dPEG (T_d = 58 °C) that included PEP, EI, and HPr. Upon starting the reaction with mannitol, a low initial activity was found (Fig. 5*B*, ●). However, when mannitol replaced PEP in the incubation step and the reaction was started with PEP, maximal activity was found almost immediately, indicating that formation of heterodimer was nearly completed at the moment that PEP was added (Fig. 5*B*, ○). When both PEP and mannitol were omitted during the incubation of the two enzymes, and the reaction was started with these two components, a similar pattern was found as when the reaction was started with mannitol (Fig. 5*B*, ×). The similarity in activity profiles of ● and × shows that prior phosphorylation of EII^{mtl} does not affect the process of heterodimer formation as probed under the conditions used.

Finally, we have investigated the formation of heterodimers in *n*-decyl β-D-maltopyranoside (dMal), a detergent which does not form micelle clusters and experience phase separation. The specific activity of EII^{mtl} in 3 mM dMal is the same as when the enzyme is solubilized in 0.25% dPEG (T_d = 58 °C) or $C_{10}E_5$ (T_d

[2] When domains are covalently linked, we refer to them as the A, B, and C domain, and when they are produced separately, we refer to them as IIA^{mtl}, IIB^{mtl}, and IIC^{mtl}.

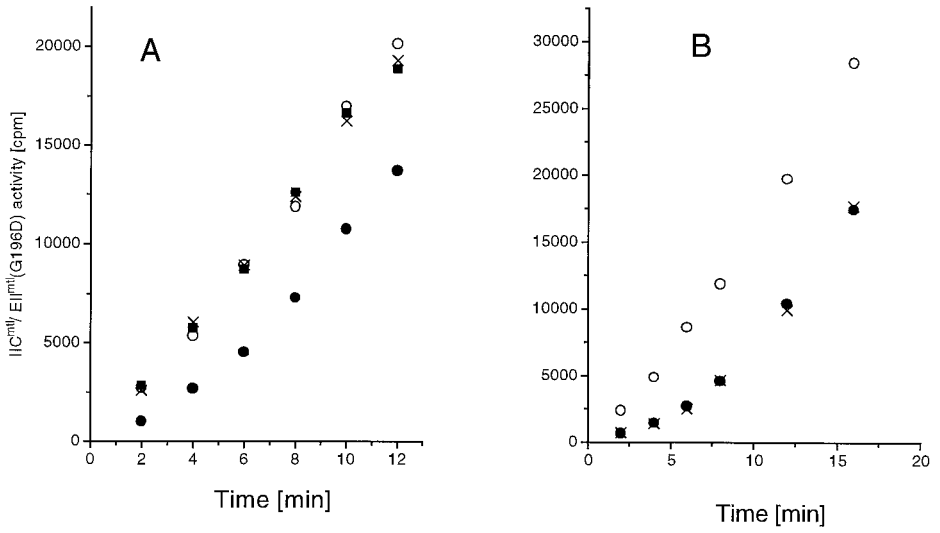

FIG. 5. **Dependence of the rate of appearance of the phosphorylation activity of EII^{mtl} (G196D)/IIC^{mtl} heterodimers on the apparent hydrodynamic radii (R_h) of the micelles and the presence of mannitol and/or PEP.** *A*, EII^{mtl} (G196D) (200 nM) and IIC^{mtl} (1.45 μM) were incubated at 30 °C for 10 min in buffer containing 0.25% dPEG, 5 mM DTT, and 25 mM Na_3PO_4 (solution 1) or 215 mM Na_3PO_4 (solution 2). ●, solution 1 was diluted 12-fold into 25 mM Na_3PO_4, pH 7.6, 5 mM DTT, 5 mM $MgCl_2$, 5 mM PEP, 0.25% dPEG, 20 μM HPr, 0.33 μM EI, and after 5 min at 30 °C, the reaction was started with 60 μM mannitol (final R_h and T_d are 3.4 nm and 58 °C, respectively). ×, solution 1 was diluted 12-fold into 215 mM Na_3PO_4, pH 7.6, 5 mM DTT, 5 mM $MgCl_2$, 5 mM PEP, 0.25% dPEG, 20 μM HPr, 0.33 μM EI, and after 5 min, the reaction was started with mannitol (final R_h and T_d are 6.4 nm and 37 °C, respectively). ○, solution 2 was diluted 12-fold into 25 mM Na_3PO_4, pH 7.6, 5 mM DTT, 5 mM $MgCl_2$, 5 mM PEP, 0.25% dPEG, 20 μM HPr, 0.33 μM EI,, and after 5 min at 30 °C, the reaction was started with 60 μM mannitol (final R_h and T_d are 3.4 nm and 58 °C, respectively). ■, solution 2 was diluted 12-fold into 215 mM Na_3PO_4, pH 7.6, 5 mM DTT, 5 mM $MgCl_2$, 5 mM PEP, 0.25% dPEG, 20 μM HPr, 0.33 μM EI, and after 5 min, the reaction was started with mannitol (final R_h and T_d are 6.4 nm and 37 °C, respectively). *B*, 20 nM EII^{mtl}(G196D) and 100 nM IIC^{mtl} were incubated at 30 °C for 10 min in 25 mM Tris·HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 5 mM PEP, 0.25% dPEG (T_d = 58 °C), 20 μM HPr, and 0.33 μM EI (●). As shown in ○, 5 mM PEP was replaced by 60 μM mannitol, whereas in ×, no PEP or mannitol was present in the incubation period. The reaction was started with 60 μM mannitol (●); 5 mM PEP (○), or 60 μM mannitol and 5 mM PEP (×).

= 44 °C), detergents in which maximal EII^{mtl} activity is observed. EII^{mtl} (G196D) (20 nM) and IIC^{mtl} (145 nM) were incubated for 5 min at 30 °C in assay buffer containing 0.25% dPEG and 140 mM Na_3PO_4 (T_d = 44 °C) (●), 3 mM dMal (○), 6 mM dMal (×), or 6 mM dMal and 140 mM Na_3PO_4 (■), and the phosphorylation assay was started with mannitol (Fig. 6). While a high activity was observed in dPEG, replacement of this detergent by dMal results in an 8–16 times lower activity, indicating that heterodimer formation was strongly suppressed in dMal presumably via suppression of homodimer dissociation.

DISCUSSION

Numerous papers have been dedicated to study the kinetics of mannitol translocation and phosphorylation of *Escherichia coli*, EII^{mtl} (20). Various biophysical techniques like radiation inactivation (21), size-exclusion chromatography (22, 23), chemical cross-linking (24), Fourier transfer infrared spectroscopy (25), and single tryptophan fluorescence spectroscopy (17) have been used to study the domain structure and oligomerization state of EII^{mtl}. While some work was carried out on proteoliposomes of EII^{mtl}, most of these studies have been performed while the enzyme was solubilized by a PEG-based detergent, especially Lubrol PX and dPEG. EII^{mtl} has been observed both as a monomer and a dimer (19, 21, 26). The crucial contacts resulting in the dimer appear to be between the hydrophobic C domains (23, 27). Stephen and Jacobson (19) found that, upon mild extraction of the enzyme from vesicles, the percentage of dimer increased with increasing ionic strength but decreased upon introduction of the PEG-based detergent, Lubrol PX, or mannitol or upon phosphorylation of the enzyme. Kinetic experiments have shown that the dimer is primarily responsible for the PEP-dependent phosphorylation and mtl/mtl-P exchange (10, 28). The formation of heterodimers is another approach used to show that EII^{mtl} dimers are functional (18, 27, 29, 30). Mutants of EII^{mtl}, each inactive by virtue of a mutation in the A, B, or C domain, could be

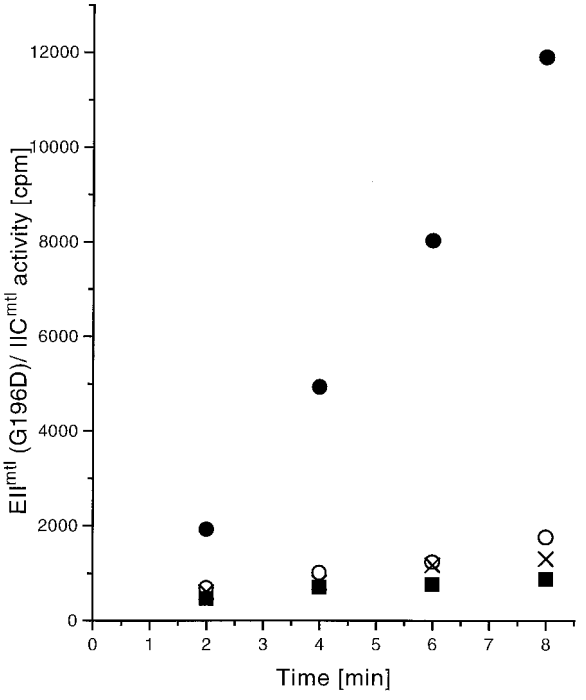

FIG. 6. **Effect of detergent on the PEP-dependent phosphorylation activity of EII^{mtl}(G196D)/IIC^{mtl} heterodimers.** 20 nM EII^{mtl} (G196D) and 145 nM IIC^{mtl} were incubated for 5 min at 30 °C in 25 mM Tris·HCl, pH 7.6, 5 mM DTT, 5 mM $MgCl_2$, 5 mM PEP, 20 μM HPr, 0.33 μM EI containing 0.25% dPEG (●) and 140 mM Na_3PO_4 (T_d = 44 °C), 3 mM dMal (○), 6 mM dMal (×), and 6 mM dMal (■) and 140 mM Na_3PO_4, respectively. The reaction was started with 60 μM mannitol.

reactivated by mixing with another mutant form carrying the mutation on another domain (see Fig. 4, *inset*). Apparently, the phosphoryl group can cross the dimer interface when proceed-

ing from the A domain, via the B domain, to mannitol bound at the C domain.

The following evidence indicates that an increased tendency of the micelles to form clusters (high R_h conditions) induces monomerization of EII^{mtl} dimers. 1) A lowering of the EII^{mtl} activity was observed with increasing R_h. 2). The specific activity of EII^{mtl}, under conditions of high R_h, increased with increasing enzyme concentration. A higher percentage of (active) dimers is expected upon an increase in enzyme concentration, due to mass action. Under conditions of minimal R_h (maximal T_d), maximum activity was observed at all EII^{mtl} concentrations monitored, indicative of a completely dimeric enzyme. The increase of specific activity which Boer *et al.* (27). observed upon addition of high concentrations of IIC^{mtl} to active EII^{mtl} can also be explained in these terms; a population of EII^{mtl} monomers were present under their specific detergent conditions, which were titrated by high concentrations of IIC^{mtl} to form active heterodimers. 3) The rates of heterodimer formation, from two homodimers, were significantly increased under conditions of high R_h due to the increased rate of dissociation of the homodimers. In line with this, the kinetics of heterodimer formation were also increased by the introduction of mannitol, a substrate known to dissociate EII^{mtl} (19).

The relationship between the EII^{mtl} activity and the T_d of dPEG or $C_{10}E_5$ was found to be similar, whether the lowering of T_d was caused by $C_{10}E_4$ or Na_3PO_4 (Fig. 1). This, and the observation that the EII^{mtl} activity at low T_d can be completely converted to the high activity found in buffer with a high T_d, supports the view that the enzyme is sensitive to changes in the micellar properties rather than the chemical composition of the detergent. EII^{mtl} exhibits maximal activity when solubilized in dPEG, $C_{10}E_5$, or $C_{10}E_6$ (not shown). The micellar size of dPEG (T_d = 58 °C), $C_{10}E_5$ (T_d = 44 °C), and $C_{10}E_6$ (T_d = 62 °C) at 30 °C, where almost no clustering of micelles is expected, differs significantly (R_h is 3.4 nm, 4.9 nm, and 2.9 nm, respectively). Apparently, the size of the micelle is not important for the monomer to dimer equilibrium. The concentration of micelles is 4 orders of magnitude higher than the enzyme concentration used in these experiments. Clustering of micelles, including the ones containing EII^{mtl}, will result in partial solvation of the EII^{mtl}-detergent micelles by other detergent micelles instead of by bulk water. This shift in solvation is expected to increase with increasing micelle cluster size. Since hydrophobic forces are involved in the formation of the EII^{mtl} dimer (10, 19), the shift to a more hydrophobic environment most likely explains the dissociation of dimers.

Recently, Boer *et al.* (18). demonstrated that the simultaneous expression of EII^{mtl} (G196D) and IIC^{mtl} in *E. coli.* resulted in cells that were able to take up mannitol. No mannitol uptake was observed if the mutants were expressed separately. This experiment showed that heterodimer formation between EII^{mtl} (G196D) and IIC^{mtl} occurs *in vivo*. Heterodimer formation also proceeds in dPEG and $C_{10}E_5$ and is facilitated by an increase of R_h (decrease in T_d). Almost no heterodimer formation was found in dMal although the wild-type enzyme is highly active when solubilized in this detergent. Therefore, PEG-based detergents are probably the best class of detergents for mechanistic investigations on EII^{mtl} since the activity is comparable with the activity for EII^{mtl} in vesicles (31), and heterodimer formation is readily achieved in these detergents.

In conclusion, a "new" detergent parameter relevant for membrane protein chemistry, *i.e.* the tendency of micelles to cluster, has been shown to control the oligomerization state of EII^{mtl}. Proper control of this variable can result in almost complete monomerization or dimerization of the protein. It provides a unique tool to study mechanistic aspects of membrane protein oligomerization.

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REFERENCES

1. Nilsson, P. G., and Lindman, B. (1983) *J. Phys. Chem.* **87,** 4756–4761
2. Zulauf, M. (1991) in *Crystallization of Membrane Proteins* (Michel, H., ed) pp. 53–72, CRC Press, Boca Raton, FL
3. Komaromy-Hiller, G., Calkins, N., and von Wandruszka, R. (1996) *Langmuir* **12,** 916–920
4. Corti, M., and Degiorgio, V. (1981) *J. Phys. Chem.* **85,** 1442–1445
5. Hayter, J. B., and Zulauf, M. (1982) *Colloid Polym. Sci.* **260,** 1023–1028
6. Corti, M., Degiorgio, V., and Zulauf, M. (1982) *Phys. Rev. Lett.* **48,** 1617–1620
7. Zulauf, M., and Rosenbusch, J. P. (1983) *J. Phys. Chem.* **87,** 856–862
8. Mitchell, D. J., Tiddy, G. J. T., Waring, L., Bostock, T., and McDonald, M. P. (1983) *J. Chem. Soc. Faraday Trans. 1* **79,** 975–1000
9. Bordier, C. (1981) *J. Biol. Chem.* **256,** 1604–1607
10. Lolkema, J. S., and Robillard, G. T. (1990) *Biochemistry* **29,** 10120–10125
11. Dooijewaard, G., Roossien, F. F., and Robillard, G. T. (1979) *Biochemistry.* **18,** 2990–2996
12. Robillard, G. T., Dooijewaard, G., and Lolkema, J. S. (1979) *Biochemistry* **18,** 2984–2989
13. van Dijk, A. A., de Lange, L. C., Bachovchin, W. W., and Robillard, G. T. (1990) *Biochemistry* **29,** 8164–8171
14. Pas, H. H., ten Hoeve Duurkens, R. H., and Robillard, G. T. (1988) *Biochemistry* **28,** 5520–5525
15. Robillard, G. T., and Blaauw, M. (1987) *Biochemistry* **26,** 5796–5803
16. Lolkema, J. S., Dijkstra, D. S., ten Hoeve Duurkens, R. H., and Robillard, G. T. (1990) *Biochemistry* **29,** 10659–10663
17. Swaving Dijkstra, D., Broos, J., Lolkema, J. S., Enequist, H., Minke, W., and Robillard, G. T. (1996) *Biochemistry* **35,** 6628–6634
18. Boer, H., ten Hoeve Duurkens, R. H., and Robillard, G. T. (1996) *Biochemistry* **35,** 12901–12908
19. Stephan, M. M., and Jacobson, G. R. (1986) *Biochemistry* **25,** 4046–4051
20. Lolkema, J. S., and Robillard, G. T. (1992) *New Compr. Biochem.* **21,** 135–167
21. Pas, H. H., Ellory, J. C., and Robillard, G. T. (1987) *Biochemistry* **26,** 6689–6696
22. Khandekar, S. S., and Jacobson, G. R. (1989) *J. Cell. Biochem.* **39,** 207–216
23. Lolkema, J. S., Kuiper, H., ten Hoeve Duurkens, R. H., and Robillard, G. T. (1993) *Biochemistry* **32,** 1396–1400
24. Roossien, F. F., van Es Spiekman, W., and Robillard, G. T. (1986) *FEBS Lett.* **196,** 284–290
25. Boer, H. (1996) *Domain Interactions in the Mannitol Transport Protein of Escherichia coli.* Ph.D. thesis, University of Groningen, The Netherlands
26. Roossien, F. F., and Robillard, G. T. (1984) *Biochemistry* **23,** 5682–5685
27. Boer, H., ten Hoeve-Duurkens, R. H., Schuurman-Wolters, G. K., Dijkstra, A., and Robillard, G. T. (1994) *J. Biol. Chem.* **269,** 17863–17871
28. Saier, M. H. (1980) *J. Supramol. Struct.* **14,** 281–294
29. van Weeghel, R. P., van der Hoek, Y. Y., Pas, H. H., Elferink, M., Keck, W., and Robillard, G. T. (1991) *Biochemistry* **30,** 1768–1773
30. Weng, Q. P., Elder, J., and Jacobson, G. R. (1992) *J. Biol. Chem.* **267,** 19529–19535
31. Lolkema, J. S., ten Hoeve-Duurkens, R. H., and Robillard, G. T. (1993) *J. Biol. Chem.* **268,** 17844–17849